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Purification and toxicity studies of stevioside from *Stevia rebaudiana* Bertoni

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Abstract

The present study was undertaken to purify the stevioside from leaves of *Stevia rebaudiana* and to determine its toxicity effects. The accumulation of stevioside was found in all parts of the *Stevia* plant that is from root to flowers with the highest content in mature leaves. A protocol has been standardized for the production of white stevioside powder from dried mature leaves of *Stevia rebaudiana* using water as solvent in the extraction and filtration procedures. The pigments and phenols in the crude extract were removed at the first step of processing. The depigmented extract was bleached and refined by celite treatment at the second level. The purity at each step was checked by HPLC using purified stevioside as standard. The end solution was spray dried into fine white stevioside powder. The final yield was estimated as 11.6%, which is equivalent to international standard. The protocol was found reliable and economical for the commercial production of stevioside as a natural sweetener. The method was filed for Indian patent no: 01436/CHE/2007. As a zero calorie natural sweetener with more potential application in the treatment of Type II diabetes, toxicological analysis of the product was done for revealing the acute oral toxicity and cytotoxicity of the product. In the oral toxicity test none of the animals showed any abnormal behaviour and all the organs were found physiologically normal at a higher dosage of 2000mg stevioside/kg bodyweight on feed trials suggesting the non-toxic nature of stevioside in rats. The *in vitro* cytotoxicity test has revealed the non cytotoxic property of stevioside at a concentration of 1.25g/L.

Keywords: Nontoxic, S*tevia rebaudiana*, purification, stevioside powder, toxicity test

Introduction

Stevia rebaudiana Bertoni (Asteraceae) is a sweet herb indigenous to Paraguay. The leaves of the plant accumulate a mixture of eight diterpene glycosides commonly called as steviosides, the concentrations of which vary quite widely on the genotype and the production environment (Brandle and Telmer, 2007). These natural products taste intensely sweet and it has been up to be 300 times sweeter than sucrose on weight basis (Richman *et al.,* 1999). As a natural non-caloric sweetener, the stevioside has been used to sweeten a variety of foods including sea foods, pickled vegetables, dessert items, soft drinks and confectionary for decades (Geuns, 2004). The leaves of the plant have been used for centuries in the treatment of diabetes as a traditional medicine. Interest has been rekindled in more recent years especially in developing countries where diet conscious consumers seek a natural non-caloric sweetener as an alternative to chemical sweeteners. Experimental studies on animals and human volunteers have indicated the antihyperglycemic and insulinotropic effects of steviosides (Chan *et al.,* 1998; Hsieh *et al.*, 2003; Jeppesen *et al.*, 2002). Stevioside, as a zero caloric natural sweetener with the highest rate of sweetness, ambiguity still persists, on its toxicological properties whether non-toxic or not. However, several studies support the non-toxic effect of stevioside highlighting its positive functions on cell metabolism (Gregersen *et al.*, 2004; Abdullah *et al.*,

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2004). As a secondary metabolite reserve accumulated in the leaves of *Stevia,* maximum concentration was found in mature leaves ie, 10-12% on dry weight basis (Brandle and Rosa, 1992). There are a number of patented refining processes registered internationally, for the production of stevioside commercially. Methanol has been used in most extraction and purification process as the solvent, presumably to improve extraction efficiency. The use of methanol even though it is all removed from the final product appears to be the reason for not classifying *Stevia* extracts as natural and GRAS (generally regarded as safe) by US FDA (United State Food and Drug Administration). Therefore the present study was undertaken to develop a protocol for the purification of steviosides from *Stevia* leaves using water for extraction and filtration procedures. The study also aims to evaluate the oral toxicity and cytotoxicity of the steviosides.

Materials and Methods

Plant material - Healthy plants of *Stevia rebaudiana*, maintained in the experimental *Stevia* garden of SIBB R&D were selected for the study. All parts of the *Stevia* plant viz. leaves, buds, stem, roots and flowers were tested for estimating the stevioside content. The aqueous extract was prepared from the dried parts and estimated spectrophotometrically. For the purification, the fresh mature leaves were collected from healthy *Stevia rebaudiana* plants prior to flowering.

Isolation and Purification of stevioside

Drying of leaves - The leaves were kept in a hot air oven at 70-80°C for 8hrs for drying. This thermal treatment for a long duration helped to maintain the quality of the leaf sample from over drying as used in conventional drying ie, at 110°C for 3 hr.

Extraction - The dried leaves (1kg) were boiled with water (5L) for 3 hr. The extract was filtered through single layered cheese cloth. The stevioside content in the crude extract was quantified spectrophotometrically at 200 nm using stevioside as standard (NuNaturals). The stevioside content was estimated at regular time intervals of 30 min for optimizing the extraction time.

Purification

Step I - The crude green extract was treated with 10% activated charcoal and stirred well for 20 minutes for the first level of purification. The extract was filtered and the residual charcoal was again mixed with boiling water for 10 min and filtered to recover the whole stevioside content from charcoal. The process was repeated thrice for the maximum recovery. The filtrates were mixed for the second step of purification. Stevioside content of each recovery was quantified by reading the optical density at 200 nm. The content of total phenols, chlorophyll and carotenoids were determined to check the level of purity of the stevioside extract (Sukran *et al.,* 1998).

Step II - The yellow colored extract was treated with 5% celite and stirred for 20 min. The celite was initially moisturized with water before the treatment of the extract and the mixture was filtered. The celite residue was again mixed with boiling water for 10 min for further filtration. The colorless stevioside extract recovered after celite treatment was centrifuged at 12,000 rpm for 15 min and the clear aqueous solution was collected and stored. At each step of the purification, the parameters such as pH, phenol and pigment content were checked. The purity of stevioside was tested by HPLC and compared with the crude extract (Minne *et al.,* 2004).

Micro spray drying - The refined stevioside solution was spray dried using the following conditions: inlet temperature 180°C; outlet temperature 95°C; pump pressure 500 psi. Air assisted twin jet nozzle with a diameter of 0.75 mm was used for getting white stevioside powder.

Acute oral toxicity study

Animal preparation - The animals were collected from the laboratory division of animal science, Sree Chitra Tirunal Institute for Medical Sciences and Technology (SCTIMST), Thiruvananthapuram. Randomly selected healthy, adult, active, female, not previously used wistar rats of 179 $\pm$ 5 g of body weight were used for the study. They were housed in polypropylene cages with paddy husk bedding and stainless steel top. Identification was given in individual cage tags with picric acid mark on animals. The rats were maintained by natural climate with natural day and

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night cycles and given standard commercial diet and clean water *ad libitum*. The animals were acclimatized for 5 days prior to the test. The protocol for the test was done in accordance with Organization for Economic Corporation and Development (OECD) guideline for testing.

Sample preparation - The white stevioside powder was dissolved in aquaguard water with a concentration of 5 g/L as stock. It was further diluted as per the dosage of the test study. The rats were divided into two groups with five rats in each group. The grouping was done based on the dosage range of the test sample stevioside ie, 300mg/kg body weight as lower concentration for the one group of rats and 2000 mg/kg body weight as higher concentration for the other group baed on OECD guidelines with document No. 420. For the sighting study, one animal from each group was selected. A dosage of 300 mg/kg body weight stevioside powder in aquaguard water was given to animal of the first group and 2000 mg/kg body weight was given to the animal of the second group. The test solution was loaded in a syringe after thorough agitation. The administration of solution containing test material was given to rats using a gastric needle. Same concentrations were followed to the remaining rats (four) of each group ie, 300mg/kg and 2000 mg/kg**.** The volume of injection was 1 mL/ 100 g body weight. Overnight fasting prior to dosing and 3 to 4 hr after the test was done.

Post observation of the animals - All the animals were observed at 30 min, periodically during the first 24 hr and thereafter daily for 14 days. Appearance of skin, fur, eyes, respiratory, autonomic and central nervous system were observed during these days. At the end of 14 days all the animals were sacrificed humanely and observed for gross pathology.

Gross pathology - Gross pathological examinations were carried out and the following organs were examined at necropsy - lungs, oesophagus, trachea, pharynx, larynx, thyroid, parathyroid, thymus, heart, liver, spleen, stomach, small intestine, rectum, ovaries, uterus, cervix, vagina and vulva, kidneys, ureters, adrenals and urinary bladder.

In vitro cytotoxicity test

In vitro cytotoxicity test was done with stevioside extract in mammalian fibroblast cell line culture (ATCC, L-929) for the duration of three days. Extract was prepared by incubating test sample with minimum essential medium (MEM) supplemented with Fetal Bovine Serum culture medium at 37 ± 2ºC for 24 ± 2 hr. The extraction ratio administrated for the study was 5g/L, 2.5 g/L and 1.25 g/L with proper dilution. Different dilutions of extracts of test sample, ultra high molecular weight polyethylene as negative control and dilute phenol as positive control in triplicates were placed on sub-confluent monolayer of L-929 cells. After incubation of cells with extracts of test sample and controls at 37 ± 2ºC for 24 ± 1h cell cultures were examined microscopically for cellular response. Cellular response was scored as 0, 1, 2, 3 & 4 according to none, slight, mild, moderate and severe.

Results and Discussion

White stevioside powder

The accumulation of steviosides in different parts of the *Stevia* plant was determined spectrophotometrically at 200 nm. As a secondary metabolite, the concentration of steviosides showed wide variation in the plant system with maximum concentration in leaves and minimum in roots. Since chloroplast has been identified as the sub- cellular site of kaurene, the precursor of stevioside synthesis, the accumulation of stevioside more in leaves than other parts did not require any further clarification (Brandle and Telmer, 2007). In the vegetative parts, mature leaves showed higher deposition than the tender leaves indicates the correlation of stevioside synthesis and the growth of the plant. The sweetening property of the raw mature leaves of this splendid plant was estimated as 20 to 30 times sweeter than sucrose (Brandle *et al.,* 1998). Hence the mature leaves of the plant were used for the whole purification process. On dry weight basis, the average content of steviosides in *Stevia* leaves was reported as to 10 to 12% (Geuns *et al.,* 1992). The crude leaf extract was deep green in color due to the presence of pigments and other soluble components. Figure 1 is the chromatogram of the crude extract showing several peaks along with a major peak for stevioside at the retention time 2.1 min. In the first step of purification of treating the extract with activated charcoal, the deep green color of the extract was reduced to a transparent light yellow. The variations observed in the concentration of phenols

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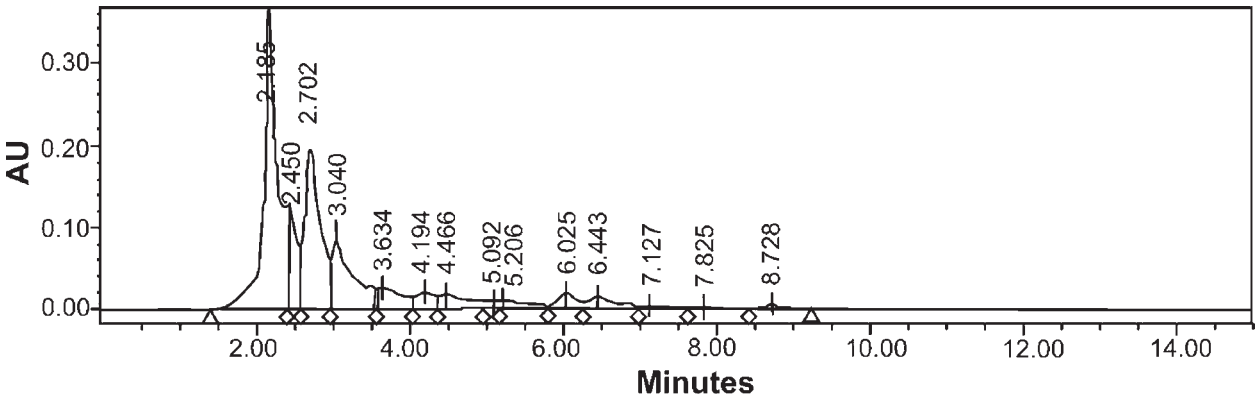


Fig.1: Chromatogram of crude leaf extract by HPLC

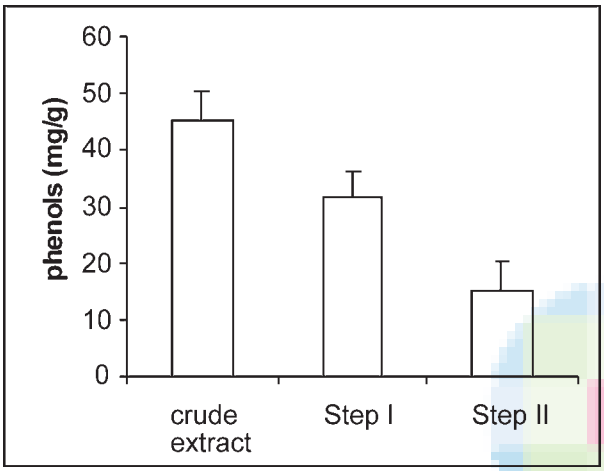


Fig.2: Total phenolic content in the extracts after each step of purification

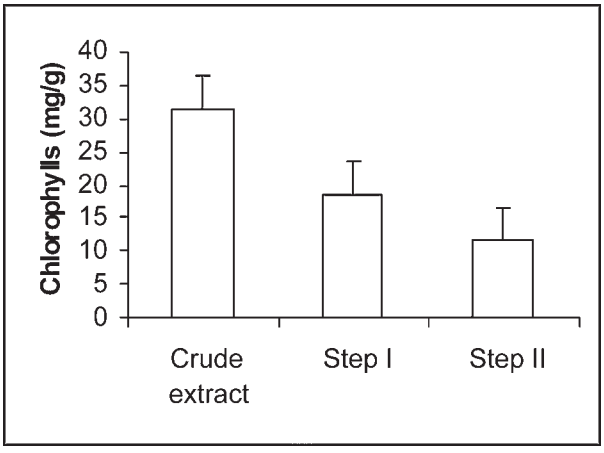


Fig.3: Total chlorophyll content after each step of purification

and total chlorophylls and carotenoids during purification were displayed in figures 2, 3 & 4. A drastic depletion was noticed in phenol and chlorophyll content indicating the progressive level of purification. The disappearance of the color

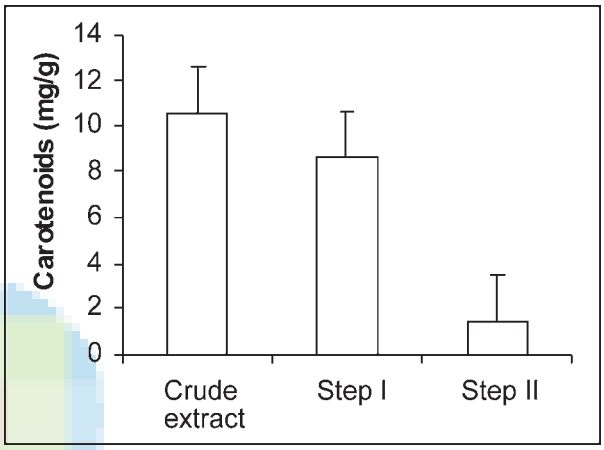


Fig.4: Level of carotenoids during purification

suggests the removal of pigments and phenols by charcoal treatment. The total content of stevioside estimated in the crude extract showed a two-fold reduction revealing the purity of the end product. The light yellow colored extract was further bleached and refined by treating the extract with celite. After the celite treatment, the filtrate appeared as colorless with pH 6.0. The HPLC profile of purified stevioside extract showed a prominent single peak similar to that of the standard (Fig.5&6). At each step of purification, the presence of stevioside in the filtrate was checked at 200 nm until the OD becomes negligible. The colorless stevioside extract was further centrifuged for removing all the soluble impurities prior to microspray drying. The final concentration of stevioside was 11.6%.

The aqueous stevioside solution was spray dried at 180°C inlet temperature and 95°C outlet temperature with 500 psi pump pressure. The fine

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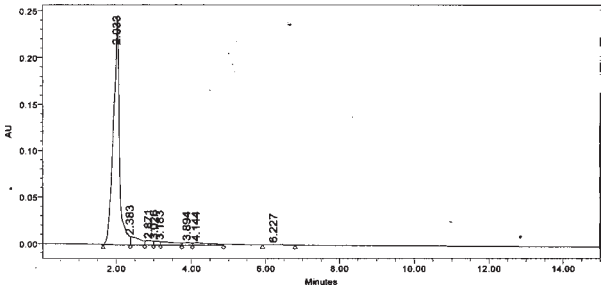


Fig.5: Chromatogram of the purified stevioside after charcoal and celite treatment

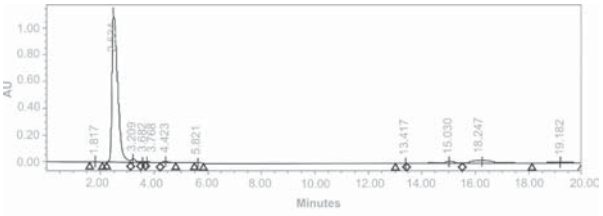


Fig.6: Chromatogram of the standard stevioside powder



Fig.7: The fine powdered white stevioside.

powdered white stevioside with a moisture content of 3.9% was collected (Fig.7). The result clearly demonstrates the feasibility of producing white stevioside powder from dried mature leaves. Since the percentage of recovery of stevioside is equivalent to international refining processes, this protocol would be affordable to farmers in both small scale and large scale conditions.

Toxicity test of stevioside

Acute oral toxicity - All the experimental animals were observed normal during the observation period. The animals were fed with stevioside at a concentration of 300 mg/kg bodyweight and 2000 mg/kg body weight respectively. No changes on skin, fur, eyes, respiratory, autonomous and central nervous system were observed during these days. None of the animals showed any abnormal behaviour like tremors, convulsion, salivation, diarrhoea, lethargy, sleep or coma. There was no loss in body weight. At the end of the observation period all the animals were sacrificed and gross pathology was carried out. There was no abnormality detected in gross necropsy in the test animals and all the organs were found normal. Since there was no death of the dose of 2000 mg/kg body weight, the test sample stevioside is found to be non-toxic in rats, at those experimental conditions stimulated. Based on these observations, as per globally harmonized classification system for chemical substances and mixtures, stevioside powder can be classified as an unclassified, non-toxic compound. Despite the use of stevioside as a zero calorie sugar substitute, this compound received more clinical attention against Type II diabetes due to its insulinotropic and antihyperglycemic properties. So toxicity studies to assess the safety of this compound for human consumption have become a pre-requisite for the promotion of the product. Stevioside as a molecule of clinical interest, Geuns (2003b) has revealed a very low toxicity of stevioside in acute toxicity studies, and in the study, he concluded that *Stevia* and stevioside are safe when used as a sweetener. An acceptable daily intake (ADI) of 7.9mg stevioside/kg body weight was calculated for human consumption (Xili *et al.,* 1992). However, this ADI should be considered as a minimum value as the authors did not test concentrations of stevioside higher than 7.9 mg/kg body weight. The easy uptake of stevioside by the gastro-intestinal tract of rodents and pigs was demonstrated by animal experiments and the data conclusively support the absorptive transport of stevioside and its complete conversion in the colon. No stevioside could be detected in the blood of the animals (Koyama *et al.,* 2003b; Geuns, 2003b).

Cytotoxic effect - Cytotoxic property of the stevioside powder was tested by incubating serum based stevioside medium on sub-confluent monolayer of mammalian cell line culture cells of L-929 strain. The test method was done based on test on extracts ISO 10993-5, 1999. The test material stevioside powder was dissolved in culture medium containing serum at three different concentrations viz. 5g/L; 2.5g/L and 1.25g/L.

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Three dilutions of the test sample, negative control (without test sample) and positive control (phenol solution) in triplicate were used for the test. The extract of the stevioside powder showed severe cytotoxic response to fibroblast cells at a concentration of 5 g/L, mild cytotoxic at 2.5 g/L and non-cytotoxic at 1.25g/L. Extracts of negative control gave non-cytotoxic response and positive control gave severe cytotoxic response as expected. It is obvious from the data that stevioside was non-cytotoxic at a concentration of 1.25 g/L. The quantity of purified stevioside at a rate of 1.25 g/L is equivalent to 250-350 g/L of sucrose since stevioside is 200 to 300 times sweeter than sucrose. Hence the consumption of stevioside will not cross the limit of non toxic level when considered as a food supplement. So the non-cytotoxic limit of stevioside noticed from the *in vitro* cytotoxocity test will be more than enough to satisfy the daily requirement of an adult human being. Recent experimental studies of steviosides conducted on animals and human volunteers have provided evidence that steviosides are not genotoxic *in vitro* and *in vivo* and non-carcinogenic (Genus *et al.,* 2006; Koyama *et al.,* 2003a). On the basis of the present data, it may be concluded that stevioside is a potential natural sweetener with more ideal qualities than sucrose. However, further experimental trials are required in human volunteers to establish non toxic and therapeutic values of this bioproduct.

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